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TITLE: Assessment of Pubertal Development and Thyroid Function in Juvenile Male CD® (Sprague-Dawley) Rats After Exposure to Selected Chemicals Administered by Gavage on Postnatal Days 23 Through 52/53

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PROPOSED STUDY IN-LIFE DATES: May 2002 - July 2002 (Component 1)
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AMENDMENTS:

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1.0 OBJECTIVE AND BACKGROUND

The objective of this study is to quantify the effects of environmental compounds on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. This assay detects compounds that display antithyroid, estrogenic, androgenic, antiandrogenic [androgen receptor (AR) or steroid enzyme mediated] activity, or alter follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, growth hormone (GH), or hypothalamic function.

The Food Quality Protection Act of 1996 required the EPA to develop and implement a screening program for determining the potential in humans for estrogenic (and anti-estrogenic) effects from pesticides. This program has been expanded on the advice of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to include androgenic (and anti-androgenic) effects and effects from thyroid-hormone (TH)-like (and anti-TH) substances.

The EDSTAC, assembled by the EPA in 1996, recommended the use of a female 20-day pubertal assay with thyroid to evaluate test materials for effects on the thyroid, hypothalamic-pituitary-gonadal (HPG) axis, aromatase and estrogens (and/or other test materials) that are only effective orally, or after a dosing duration longer than that used in the uterotrophic assay (EDSTAC Report, 1998, Vol. 1, Chapter 5, p. 5-26). EDSTAC also recommended, as an alternate assay to be evaluated, the male 20-day thyroid/pubertal assay in rodents (EDSTAC, 1998, Vol. 1, Chapter 5, p. 5-30).

The EDSTAC discussion on the usefulness of the male pubertal assay and its endpoints included the following:

> "This assay detects androgens and antiandrogens *in vivo* in a single stage apical test. "Puberty" is measured in male rats by determining age at PPS (preputial separation). Animals are dosed by gavage beginning one week before puberty (which occurs at about 40 days of age) and PPS is measured. Androgens will accelerate and antiandrogens and estrogens will delay PPS. The assay takes about 3 weeks, and allows for comprehensive assessment of the entire endocrine system in one study (10 per group, selected for uniform body weights to reduce variance). The animals are dosed daily, seven days a week, and examined daily for PPS. Dosing continues until 53 days of age; the males are then necropsied. The body, heart (thyroid), adrenal, testis, seminal vesicle plus coagulating glands (with fluid), ventral prostate, and levator ani plus bulbocavernosus muscles (as a unit) are weighed. The thyroid is retained for histopathology and serum is taken for T4, T3, and TSH. Testosterone, LH, prolactin, and dihydrotestosterone analyses are optional. These endpoints take several weeks to evaluate and are affected not only by estrogens but by environmental antiandrogens, drugs that affect the hypothalamic-pituitary axis (Hostetter and Piacsek, 1977; Ramaley and Phares, 1983), and by prenatal exposure to TCDD (Gray et al., 1995a; Bjerke and Peterson, 1994) or dioxin-like PCBs (Gray et al., 1995b). In contrast to these other mechanisms, only peripubertal estrogen administration accelerates this process in the female and delays it in the male. Preputial separation in the male rodent is easy to measure and this is not a terminal measure (Korenbrot et al., 1977).
>
> Age and weight at puberty, reproductive organ weights, and serum hormone levels can also be measured. Delays in male puberty results form exposure to both estrogenic and antiandrogenic

chemicals including methoxychlor (Gray et al., 1989), vinclozolin (Anderson et al., 1995a), and p,p'DDE (Kelce et al., 1995). Exposing weanling male rats to the antiandrogenic pesticides p,p'DDE or vinclozolin delays pubertal development in weanling male rats as indicated by delayed preputial separation and increased body weight (because they are older and larger) at puberty. In contrast to the delays associated with exposure to estrogenic substances, antiandrogens do not inhibit food consumption or retard growth (Anderson et al., 1995b). Antiandrogens cause a delay in preputial separation and affect a number of endocrine and morphological parameters including reduced seminal vesicle, ventral prostate, and epididymal weights. It is apparent that PPS is more sensitive than are organ weights in this assay. In addition, responses of the HPG are variable. In studies of vinclozolin, increases in serum LH were a sensitive response to this antiandrogen, whereas serum LH is not increased in males exposed to p,p'DDE during puberty (Kelce et al., 1997). Furthermore, a systematic review of the literature indicates that the sex accessory glands of the immature intact male rat are consistently more affected than in the adult intact male rat.

In summary, preputial separation and sex accessory gland weights are sensitive endpoints. However, a delay in preputial separation is not pathognomonic for antiandrogens. Pubertal alterations result from chemicals that disrupt hypothalamic-pituitary function (Huhtaniemi et al., 1986) and, for this reason, additional *in vivo* and *in vitro* tests are needed to identify the mechanism of action responsible for the pubertal alterations. For example, alterations of prolactin, growth hormone, gonadotrophin (LH and FSH) secretion, or hypothalamic lesions alter the rate of pubertal maturation in weanling rats.

As indicated above, the determination of the age at "puberty" in the male rat are endpoints that already have gained acceptance in the toxicology community. Preputial separation in the male is a required endpoint in the new EPA two-generation reproductive toxicity test guideline. In this regard, this assay would be easy to implement because these endpoints have been standardized and validated and PPS data are currently being collected under GLP conditions in most toxicology laboratories. In addition, PPS data are reported in many recently published developmental and reproductive toxicity studies (i.e., see studies from R.E. Peterson's, J. Ashby's, R. Chapin's and L.E. Gray's laboratories on dioxins, PCBs, antiandrogens, and xenoestrogens).

Sex accessory gland weights in intact adult male rats also can be affected directly or indirectly by toxicant exposure. The HPG axis in an intact animal is able to compensate for the action of antiandrogens by increasing hormone production, which counteracts the effect of the antiandrogen on the tract (Raynaud et al., 1984; Edgren, 1984; Hershberger, 1953)." (EDSTAC, 1998, Vol. 1, Chapter 5, pp. 5-30 through 5-32).

Based on the EDSTAC's recommendations, one of the assays that the EPA has proposed to validate as a potential alternative for other assays in the Tier 1 battery in an endocrine disruptor screening program is a male pubertal assay (see FR Vol. 63, No. 248, pp. 71541-71568, December 28, 1998). This assay is the most comprehensive assay in the proposed Tier 1 battery of assays, as it is capable of detecting substances that alter thyroid function, that are aromatase inhibitors, androgens, anti-androgens, and that are agents which interfere with the hypothalamus-pituitary-gonadal axis. Results from other shorter assays and/or with the use of ip injection as the route of administration, have also been reported (O'Connor et al., 1996, 1999).

EPA has already tested the response of the pubertal assays to a variety of chemicals, but at only one dose per chemical (Rocca and Borst, 2000; Rocca and Pepperl, 2000a,b,c). EPA is in

the process of testing the response of two chemicals (vinclozolin and methoxychlor) at multiple doses to determine the sensitivity of the assays to subtle effects of estrogens and antiandrogens.

Although the experiments that have been completed or are in progress are believed to be sufficient to demonstrate the usefulness of these pubertal assays for a wide variety of chemicals, EPA feels that additional multiple-dose studies across an array of chemicals will provide greater confidence in the reliability and relevance of the assays. Therefore, EPA has decided to test ten additional chemicals that have various modes of action. Some chemicals will be tested in only males, others will be tested only in females, and some will be tested in both sexes.

2.0 MATERIALS AND METHODS

2.1 Test Substances

2.1.1 Atrazine

CAS Number: 1912-24-9

2.1.2 Propylthiouracil

CAS Number: 51-52-5

2.1.3 Ketoconazole

CAS Number: 65277-42-1

2.1.4 Linuron

CAS Number: 330-55-2

2.1.5 p,p'DDE

CAS Number: 72-55-9

2.1.6 Procymidone

CAS Number: 32809-16-8

NOTE: All additional information on the test chemicals (e.g., supplier, batch/lot number, purity, appearance, molecular formula, molecular weight, storage conditions of bulk chemical, and of dosing suspensions, etc.) will be added to the protocol by amendment.

2.2 Chemical Safety and Handling

See MSDSs of all chemicals in Attachment.

2.3 Dose Formulation and Analysis

The dosing suspensions will be prepared at a frequency determined by stability tests performed prior to the start of the study. Suspensions will be prepared at Battelle Chemical Repository, Sequim, WA, and stored in wide-mouth, amber bottles. They will be shipped via 24-hour express delivery and logged into the Materials Handling Facility prior to transfer to the Reproductive and Developmental Toxicology Laboratory for dosing. The test materials will be suspended in Mazola® corn oil (CAS No. 8001-30-7), with the concentration determined by the following formula:

$$\text{Concentration (mg / ml)} = \frac{\text{Dose per time (mg / kg)}}{\text{Dosage volume per time (5.0 ml / kg)}}$$

An aliquot of each dose level per formulation will be analyzed by Battelle. The dosing bottles will be identified at RTI by a five-digit random number Rx code, and a color code. Personnel, other than the Laboratory Supervisor, Project Toxicologist, and Study Director, will not be informed of the test chemicals or formulation concentrations until all laboratory work is completed (i.e., the study technicians will be "blind" for chemical and dose). Aliquots from the dosing bottles will be collected on the first day of dosing (postnatal day [pnd] 23) and on the first pnd 30, 37, 44, and 51, and will be shipped to Battelle Chemical Repository, Sequim, WA, for analysis.

2.4 Animals

2.4.1 Species and Supplier

The proposed test animals will be the Sprague Dawley Derived Outbred Albino Rat Crl:CD®(SD) IGS BR supplied by Charles River Laboratories, Inc., Raleigh, NC.

2.4.2 Live Animals and Species Justification

The use of live animals has been requested by the Sponsor. Alternative test systems are not available for the assessment of effects of chemicals on reproduction and development in intact mammals for determining the potential risk for humans from endocrine-mediated effects of pesticides and other chemicals. The Charles River CD® rat has been the subject of choice on reproductive and developmental toxicology contracts at RTI since 1976, and has been used for other reproductive toxicology studies with this test material. Large historical data bases for reproductive performance and prevalence of spontaneous malformations in control rats are

available from studies conducted at RTI (currently based on over 300 control litters) as well as from the supplier (Charles River, 1988). This study does not unnecessarily duplicate any previous study.

2.4.3 Total Number, Age, and Weight

For each seven group component of this study (see Table 1), 20 timed-pregnant female rats (designated the F0 generation) will be purchased for this study, at ten to 12 weeks of age upon arrival. They will arrive at RTI on gestational day (gd) 14 (based on the vendor's designation of the day of insemination as gd 1), which is gd 13 (based on the performing laboratory's designation of the day of insemination as gd 0). One hundred five (105) offspring male rats, designated the F1 generation, will be placed on study at weaning (pnd 21), weighing approximately 48-55 grams (i.e., seven groups of 15 F1 weanling males each).

2.4.4 Quality Control

The shipment of pregnant F0 females will be quarantined on arrival, and quality control evaluation will be initiated within one day after receipt. Within one day after receipt, two female rats will be chosen from the shipment, sacrificed, and blood collected for assessment of viral antibody status. Heat-inactivated serum will be sent to BioReliance (Rockville, MD) for their Level 1 Rat Antibody Screen. The viral screen will consist of evaluation for the presence of antibodies against the following: Toolan H-1 virus (H-1), Sendai virus, Pneumonia virus of mice (PVM), rat coronavirus/ sialodacryoadenitis (RCV/SDA), Kilham rat virus (KRV), CAR Bacillus, and Mycoplasma pulmonis (*M. Pul*.). In addition, fecal samples from representative animals will be externally examined for intestinal parasites.

2.4.5 Sentinels

After the selection of F1 weanling study males, four unselected male rats (or, if necessary, one to four remaining females) will be randomly selected, eartagged, and designated as sentinels. They will be singly housed in the study room(s) with feed and water available *ad libitum* (as described below). They will be examined once daily by cageside observation for morbidity or mortality at the same time as the clinical observations or morbidity/mortality checks for the study animals. The clinical condition of sentinel animals will be recorded only in the event that an animal is moribund or found dead. If a sentinel animal is terminated moribund, blood will be collected at termination and serum samples frozen. During the F1 male necropsies, the surviving sentinel males (and/or females) will be terminated, blood samples collected, and serum samples prepared. All sentinel serum samples will be submitted to BioReliance (Rockville, MD) for serological evaluation (see above section on Quality Control).

2.4.6 Quarantine

The initial F0 timed-pregnant females will be quarantined for approximately one week (gd 13-20), with the prior concurrence of the RTI Animal Research Facility (ARF) veterinarian. They will be observed daily for general health status and ability to adapt to the ARF husbandry conditions. They will be released from quarantine, if suitable for use (based on QC results), by the attending ARF veterinarian or his designate.

2.5 Animal Husbandry

2.5.1 Housing, Feed, and Water

During the quarantine period, animals will be randomly assigned to cages. Pregnant and lactating F0 females and F1 male postweanlings will be singly housed in solid-bottom, polycarbonate cages (8"x19"x10.5") fitted with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ). Sani-Chip® cage bedding (P.J. Murphy, Forest Products, Inc., Montville, NJ) will be used in all cages. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) and tap water from the Durham, NC water system, in plastic bottles with stainless steel sipper tubes, will be available *ad libitum* for the pregnant F0 females during quarantine, for the F0 females during the rest of gestation and lactation, and for the retained F1 males. The analysis of the rodent chow for chemical composition and possible chemical contamination, and analysis of Durham City water will be provided by the suppliers and maintained in the study records. It is anticipated that contaminant levels will be below certified levels for both feed and water and will not affect the design, conduct, or conclusions of this study. Rat chow will be stored at approximately 60-70°F, and the period of use will not exceed six months from the milling date. At all times, animals will be housed, handled, and used according to the NRC Guide (NRC, 1996).

2.5.2 Environmental Conditions

Environmental conditions in the ARF will be continuously monitored, recorded, and controlled during the course of the study by an automated system (Siebe/Barber-Colman Network 8000 System with Version 4.4.1 Signal® software (Siebe Environmental Controls (SEC)/ Barber-Colman Company, Loves Park, IL). Animal rooms used for this study will be maintained on a 12:12 hour light:dark cycle. Target conditions for temperature and relative humidity in the animal rooms will be between 64-79°F (18-26°C) and 30-70%, respectively, with 10-15 air changes per hour (NRC, 1996). Temperature and/or relative humidity excursions will be documented in the study records and the final report.

2.5.3 Animal Identification

All F0 maternal rats will be individually identified by ear tag after arrival at RTI. All selected study weanling F1 males will also be uniquely identified by eartag at weaning, as well

as receiving a female study number. All data generated during the course of this study will be tracked by these numbers.

2.5.4 Limitation of Discomfort

Some postweanling toxicity may be caused by exposure at the high doses of each test material. Discomfort or injury to animals will be limited, in that if any animal becomes severely debilitated or moribund, it will be humanely terminated by CO_2 inhalation. All necropsies will be performed after terminal CO_2 asphyxiation. F1 pnd 4 culled pups will be euthanized by decapitation and discarded.

3.0 EXPERIMENTAL DESIGN

3.1 Study Design

The study will be conducted in two components. Each component will consist of two dose groups per test material (and three test materials) and one vehicle control group, each group comprised of 15 weight-matched F1 male weanlings for each of the two components of this study. The F1 study males will be dosed by gavage once daily for 31-32 consecutive days (pnd 22 to pnd 52 or 53). Table 1 presents the study design and target doses of the test chemicals. A graphical representation of the study design is presented in Figure 1 below.

Tentative Study Dates[a] (to be added to the protocol by amendment)

F0 timed-pregnant females arrive at RTI:
Parturition of F1 offspring (pnd 0):
Weaning of F1 offspring (pnd 21):
Sacrifice of F0 dams:
Dosing (pnd 23 - pnd 52/53):
Sacrifice of F1 males (on pnd 52 or 53):
Submission of nonaudited draft final report:
Submission of audited draft final report:

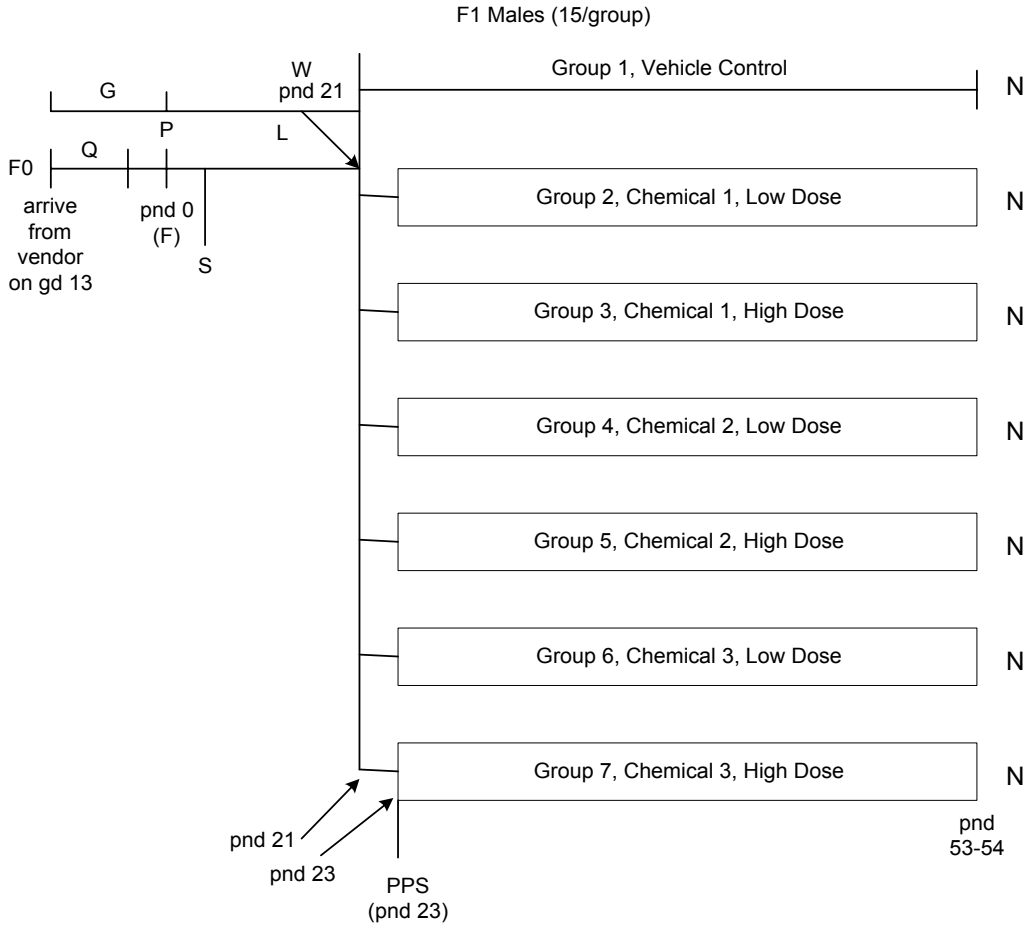
[a] The end dates are tentative and will depend on the duration of gestation and lactation of the F0 dams with F1 offspring.

Table 1. Study Design and Target Doses

Group No.	No. F1 Males	Chemical	Dose (mg/kg/day)	Concentration (mg/ml)	Dose Volume (ml/kg)
COMPONENT 1					
1	15	–[a]	0	0.0	5.0
2	15	Atrazine	75	15.0	5.0
3	15		150	30.0	5.0
4	15	Propylthiouracil	2	0.4	5.0
5	15		25	5.0	5.0
6	15	Procymidone[b]	50	10.0	5.0
7	15		100	20.0	5.0
COMPONENT 2					
8	15	Linuron	50	10.0	5.0
9	15		100	20.0	5.0
10	15	p,p'-DDE	50	10.0	5.0
11	15		100	20.0	5.0
12	15	Ketoconazole	50	10.0	5.0
13	15		100	20.0	5.0
14	15	–[a]	0	0.0	5.0

[a] corn oil, vehicle control
[b] positive control

Figure 1. Study Design for Male Pubertal Assay



KEY:

——— No exposures to F0 dams or F1 offspring during gestation or lactation

▭ Direct once daily gavage dosing of F1 males beginning on pnd 23 (see text)

Q = Quarantine (seven days, gd 13-20)

G = Gestation

P = Parturition (pnd 0)

L = Lactation

W = Wean (pnd 21) F1 pups; euthanize and discard F0 dams

F = Foster pups, if necessary, to maximize retention of F1 male pups

S = Standardize litters to ten with maximum number of F1 male pups (discard culled pups)

PPS = Acquisition of preputial separation (evaluation will begin on pnd 23)

N = Necropsy (see text)

3.2 Dosage Selection

The U.S. EPA selected the six chemicals. For five of the six, the EPA assigned doses: atrazine (affects the hypothalamus), ketoconazole (inhibits steroidogenesis), linuron (positive from prenatal exposure, negative from adult exposure), p,p'-DDE (anti-androgen), and procymidone (anti-androgen, chosen as positive control). In the two previous EPA-sponsored male pubertal assays (Rocca and Pepperl, 2000a,b), the single ketoconazole dose was 100 mg/kg/day (the high dose selected by EPA). For propylthoiuracil (affects thyroid, causes hypothyroidism), the RTI Project Toxicologist selected the two target doses, with documentation as follows.

3.2.1 Propylthiouracil (PTU)

The target doses selected were 2 and 25 mg/kg/day. Duarte et al. (2000) gavaged rats (180±10 g) daily with PTU doses of 1-50 mg/200 g body weight (equivalent to 5-250 mg/kg) for one to 30 days. The high dose, 250 mg/kg, after 30 days resulted in profound hypothyroidism (based on T3 and T4 levels). O'Connor et al. (1999) employed SD rats and ip injection (which, at least partially, circumvents first-pass metabolism in the liver) of 0, 0.025, 0.25, 1.0, or 10.0 mg/kg/day (in 0.25% methylcellulose) and reported effects on thyroid gland weight and histology and serum thyroid hormone analyses. The authors considered these parameters as "the most reliable endpoints for identifying thyroid gland toxicants in a short-duration screening battery." Radovsky et al. (2000) reported effects of PTU on the developing brain of offspring on pnd 11 from gavage administration to CD (SD) rat dams on gd 6 through pnd 10 at 38 mg/kg/day (in a developmental neurotoxicity study). In previous pubertal assays in male rats (Rocca and Pepperl, 2000a,b), the single PTU dose in both studies was 240 mg/kg/day, with excessive systemic and thyroid-mediated toxicity. All remaining studies used dosed drinking water as the route of administration of PTU.

3.3 F0 Dams and F1 Litters Prior to Weaning

3.3.1 F0 Maternal Parturition and Lactation

Beginning on gd 20, each female will be examined twice daily (a.m. and p.m.) for evidence of littering. Females who are littering at morning and afternoon checks will have this information recorded on the gestational sheet. Signs of dystocia or other signs of difficulty at parturition will be recorded. Dams that have not produced a litter by calculated gd 26 will be euthanized by CO_2 and discarded. Any dams whose whole litters are born dead or die prior to pnd 21 will be sacrificed, and the number of uterine implantation scars will be recorded.

3.3.2 Necropsy of F0 Females

On pnd 21 of each F1 litter, each F0 dam will be euthanized by CO_2 asphyxiation. The thoracic and abdominal organs will be examined for grossly evident morphological changes, and uterine implantation scars will be counted and recorded. F0 maternal carcasses and nonretained tissues will be discarded.

3.3.3 F1 Progeny

3.3.3.1 Mortality, Body Weights, and Clinical Observations. All pups will be counted, sexed, weighed, and examined as soon as possible on the day of birth (designated as pnd 0) to determine the number of viable and stillborn members of each litter. Thereafter, litters will be evaluated for survival, sex, gross observations, and body weights on pnd 4, 7, 14, and 21. Any pup which appears moribund or dies during lactation will be necropsied, when possible, to investigate the cause of death and discarded. No organs will be weighed or saved.

3.3.3.2 Standardization of Litter Sizes. On pnd 4, the size of each litter will be adjusted to ten pups, maximizing the number of male pups retained. Natural litters with ten or fewer pups will not be culled. If necessary, F1 male pups from litters with more than six males will be fostered to litters containing fewer than six males on pnd 4. All culled pups will be sacrificed by decapitation. The F0 dams will be allowed to rear their remaining F1 young to pnd 21. On pnd 21, each litter will be weaned.

3.4 Selection of F1 Weanling Males

When each F1 litter has reached pnd 21, the F1 males for each pnd 21 (wean) date will be weight ranked across litters (outliers, i.e., heaviest and lightest pups, will be eliminated from selection). The selected males will then be eartagged and distributed across the seven groups by stratified randomization (e.g., one of the seven heaviest selected males will go into each of the seven treatment groups, etc.). Of the remaining F1 males, four will be eartagged and selected as sentinels. If not enough unselected males are available, then the remaining F1 females will be used as sentinels (to obtain a total of four); see Section 2.4.5.

3.5 Treatment of F1 Weanling Males

Beginning on pnd 23, each F1 male will be dosed with one of the test materials at one of the dose levels or the vehicle control (corn oil for all chemicals). Each animal will be weighed every day prior to treatment and the body weight recorded. Treatments will be administered daily by oral gavage in 5.0 ml corn oil/kg body weight from pnd 23 and continuing through pnd 52/53. This duration of treatment is unnecessary to detect androgenic chemicals but is required for the detection of pubertal delay and antithyroid effects. Gavage dosing will use an 18-gauge gavage needle (1 inch length with 2.25 mm ball) and a 1 cc glass (disposable) tuberculin syringe for each treatment group. Xenobiotics will be administered in corn oil vehicle at a dosing

volume of 5.0 ml/kg body weight at 0700-1000 hours daily. The treatments will be administered on a mg/kg body weight basis, adjusted based on the most recent body weight, and the volume of the dose administered will be recorded each day. It is important that any dosing solutions/suspensions be well mixed to keep the chemical in suspension prior to and throughout dosing.

3.6 Observation of F1 Weanling Males

3.6.1 Clinical Observations

Clinical observations of F1 male study animals will be documented at least once daily on pnd 21 and 22 (prior to dosing period) and at least twice daily, at dosing and one to two hours postdosing, throughout the dosing period (pnd 23 through pnd 52 or 53). The examining technicians will be unaware of the test materials or of dosage levels. Observations will be made for (but not limited to):

a. Any response with respect to body position, activity, coordination, or gait
b. Any unusual behavior such as head flicking, compulsive biting or licking, circling, etc.
c. The presence of:
    1. Convulsions, tremors, or fasciculations
    2. Increased salivation
    3. Increased lacrimation or red-colored tears (chromodacryorrhea)
    4. Increased or decreased urination or defecation (including diarrhea)
    5. Piloerection
    6. Mydriasis or miosis (enlarged or constricted pupils)
    7. Unusual respirations (fast, slow, labored, audible, gasping, or retching)
    8. Vocalization

3.6.2 F1 Weanling Male Body Weights

All F1 males will be weighed in the morning on pnd 21 and 22, and every day in the morning during the dosing period on pnd 23 through pnd 52/53, for adjustment of dosing volume based on the most recent body weight. Body weights will be reported and statistically analyzed for pnd 21, 23, 30, 37, 44, 51, 52, and 53. F1 male weight gains will be calculated and analzyed for pnd 21-23, 23-30, 30-37, 37-44, 44-51, 51-52/53, and 23-52/53 (treatment period). F1 male body weights will also be recorded on the day of acquisition of preputial separation (see Section 3.6.3).

3.6.3 Acquisition of Preputial Separation

Beginning on pnd 23, each F1 study male will be examined daily for preputial separation. The appearance of partial and complete preputial separation or a persistent thread of tissue between the glans and prepuce should all be recorded if and when they occur. In addition, the body weight at complete preputial separation should be recorded. However, if a sufficient number of animals within any treatment group show persistent threads for greater than three days, a separate analysis should be conducted using the age at which the thread was first observed.

3.7 Necropsy of F1 Offspring Males

3.7.1 Terminal Blood Collection

At scheduled necropsy of the F1 males, after terminal anesthesia (CO_2 asphyxiation), the males will be weighed and the maximum amount of blood will be taken by external cardiac puncture and placed in a labeled tube. The blood will be allowed to clot and centrifuged under refrigeration at approximately 1400 x g for approximately ten minutes. The resulting serum will be subdivided into two aliquots and frozen at approximately -20°C:

a. One milliliter from each animal for analysis of thyroxine (T4) and thyroid stimulating hormone (TSH) at RTI.

b. Remaining serum from each animal shipped frozen, for possible subsequent analyses, to:

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RTD, NHEERL, US EPA
Research Triangle Park, NC 27711
Cooper.ralph@epa.gov
Phone: 919-541-4084 Fax: 919-541-5138

3.7.2 Gross Necropsy and Organ Weights

Each F1 male offspring, after blood is collected (see Section 3.7.1), will be subjected to a gross necropsy. The thoracic and abdominal organs and cavities will be examined and any abnormalities documented. The following organs will be dissected out and weighed: paired testes, paired epididymides, prostate (intact and separated into ventral and dorsolateral lobes), seminal vesicles with coagulating glands (and fluid), levator ani plus bulbcavernosus muscle complex, and thyroid (taken with attached portion of trachea, weighed after fixation and removal of the tracheal portion). Optional organs to be weighed, if warranted, include: liver, paired kidneys, adrenal glands (paired), and pituitary (fixed).

All organs will be weighed to the nearest 0.1 mg. Adrenal glands, if taken, will be weighed immediately (to minimize drying out of tissues). The pituitaries will be weighed after fixation of the head minus the lower jaw, and the thyroid will be weighed after fixation and removal of the attached trachea.

During necropsy, care must be taken to remove mesenteric fat with small surgical iris scissors from these tissues such that the fluid in the sex accessory glands is retained. Small tissues such as the adrenals, as well as tissues that contain fluid, should be weighed immediately to prevent partial drying prior to weighing.

3.7.3 Histology and Pathology

One testis, one epididymis, and the thyroid with attached portion of trachea from each F1 male will be placed in Bouin's fixative for 24 hours (then the trachea removed from the thyroid), after which they will be rinsed and stored in 70% alcohol until embedded in paraffin. They will then be sectioned at 3-5 microns and stained with hematoxylin and eosin (H and E) for subsequent histological evaluations. Optional tissues for histopathology include the liver, paired kidneys, adrenal glands (paired), and pituitary, as indicated by altered organ weight (change of "significant magnitude"), which will be processed as above. Stained sections will be evaluated by a Board Certified veterinary pathologist for pathologic abnormalities and potential treatment-related effects. Thyroids should be evaluated for morphologic changes such as altered follicular epithelial height, the relative number and staining characteristics of colloid, the extent of thyroid vascular supply, and the density, size, and shape of the thyroid follicles. The one testis and epididymis per male will be evaluated for spermatogenesis, spermiogenesis, status of seminiferous tubules in the testis, and sperm in the epididymis, as well as the structural integrity of these organs.

4.0 STATISTICAL ANALYSES

All data for a single chemical (two doses) and concurrent vehicle control group (weaning body weight, body weights and weight gains, age and weight at preputial separation, body and organ weights at necropsy, and serum hormones) will be analyzed using either parametric ANOVA under the standard assumptions or robust regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967) which do not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960). If Levene's test indicates lack of homogeneity of variance ($p<0.05$), robust regression methods will be used to test all treatment effects. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for linear trends across dose as well as overall treatment group differences (via Wald chi-square tests). Significant overall treatment effects will be followed by single degree-of-freedom *t*-tests for exposed vs. control group comparisons, if the overall treatment effect is significant. If Levene's test does not reject the hypothesis of homogeneous variances, standard

ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in SAS® Version 6 (SAS Institute, Inc., 1989a,b; 1990a,b,c; 1996; 1997) or 8 (SAS Institute, Inc., 1999a,b,c,d,e; 2000) will be used to test for linear trend, evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group to control via Dunnett's Test (Dunnett, 1955, 1964). Standard ANOVA methods, as well as Levene's Test, are available in the GLM procedure of SAS®, and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 7.5.4 (Shah et al., 1997) or Release 8.0 (RTI, 2001). Organ weights will also be analyzed by Analysis of Covariance (ANCOVA) using the body weight at necropsy as the covariate. When statistically significant effects are observed, treatment means will be examined further using LSMeans.

The unit of comparison will be the weanling F1 male offspring on study.

A test for statistical outliers will be performed in the UNIVARIATE procedure of SAS® Version 6 (SAS Institute, Inc., 1989a,b; 1990a,b,c; 1996; 1997) or 8 (SAS Institute, Inc., 1999a,b,c,d,e; 2000) on F1 male body and organ weights. If examination of pertinent study data do not provide a plausible biologically sound reason for inclusion of the data flagged as "outlier," the data will be excluded from summarization and analysis and will be designated as outliers. For all statistical tests, $p \leq 0.05$ (one- or two-tailed) will be used as the criterion for significance.

5.0 RETENTION OF SPECIMENS AND RECORDS

All specimens and records which remain the responsibility of RTI will be retained in the RTI archives for two years at the performing laboratory's expense. Beyond two years, continued retention will be at additional cost to the Sponsor.

6.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) prepared for this study.

7.0 REPORTING

An executive summary will be prepared describing the number and strain of rats used in the study, the doses and chemicals tested, and the effects with levels of statistical significance for all endpoints. Electronic and hard copies of spreadsheets containing the raw data from all animals will be provided for each endpoint. In addition, the spreadsheet should include treatment means, standard deviation, standard error, coefficient of variation, and sample number below each endpoint. Data presented should include animal number and treatment, block and

day of necropsy (if study conducted in blocks or animals killed on pnd 52 and 53), age and weight at preputial separation, body weights at weaning, organ and body weights at necropsy, body weight change from pnd 23 to necropsy, and serum T4 and TSH. A data summary table containing the mean, standard deviation, standard error, coefficient of variation, and sample size for each treatment group should be provided for all endpoints. Organ weights may be presented after covariance adjustment for necropsy body weight, but this should not replace presentation of the unadjusted data. Summaries of any histopathologic findings with photomicrographs of significant observations will also be provided.

8.0 PERSONNEL

Study Director:	Julia D. George, Ph.D.
Project Toxicologist:	Rochelle W. Tyl, Ph.D., DABT
ARF Veterinarian:	Donald B. Feldman, D.V.M., ACLAM
ARF Manager:	Frank N. Ali, M.B.A., RLATG, ILAM
Laboratory Supervisor:	Melissa C. Marr, B.A., RLATG
Data Analyst and Reproductive Toxicity Supervisor:	Christina B. Myers, M.S.
Statistical Advisor:	Gayle S. Bieler, M.S.
Research Data Entry Assistant:	Timothy W. Wiley, B.S.
Research Biologist:	William R. Ross, B.A.
Biologists:	Vickie I. Wilson Lawson B. Pelletier, RVMT, LAT
Biological Laboratory Assistants:	Charlene N. Beauman, B.S. Marian V. Rieth, RVMT Dee A. Wenzel, RVMT, LATG Robin T. Krebs, ALAS

Endocrinology:	Patricia A. Fail, Ph.D. Carol S. Sloan, M.S. Kristi D. Vick, B.S.
Histology:	Tsai-Ying Chang, B.S. HT-ASCP
Pathology:	John C. Seely, D.V.M., ACVP (EPL, Inc.)

Additional study team members to be determined.

9.0 STUDY RECORDS TO BE MAINTAINED

Protocol and any Amendments
List of any Protocol Deviations
List of Standard Operating Procedures
Animal Requisition and Receipt Records
Quarantine Records
Temperature and Humidity Records for the Animal Room(s)
Animal Research Facility Room Log(s)
Durham City Water Analysis (analyzed monthly, reported annually)
Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results
Dosage Code Records Containing Five-Digit Rx Code, Color Code, and Concentration
F0 Mating Records from Vendor
F0 Maternal Gestational and Lactational Records
Dose Formulation Receipt and Use Records
F1 Male Distribution into Groups
F1 Male Dosing Forms

F1 Male Postwean Dosing Period:	Body Weights
	Clinical Signs
	Acquisition of Preputial Separation

F1 Male Necropsy Records: Body weight, organ weights, gross observations, required (and optional, if done) organ histopathology, TSH and T4 serum levels

Statistical Analysis Records

Histopathology Report

Serum Thyroid Hormone Analyses (T4, TSH)

Correspondence

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ATTACHMENT

Material Safety Data Sheets (MSDSs)